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ADAM10 is the Physiologically Relevant, Constitutive Alpha-Secretase of the Amyloid Precursor Protein in Primary Neurons

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1st Editorial Decision 17 November 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see referee 3 is very positive about the study and would support its publication here. Referee 1 and referee 2 feel strongly that the physiological significance of your findings would need to be addressed in more depth using primary neurons. Taking into consideration all these thoughts including the concerns regarding the conceptual advance provided by the study expressed by referee 2 I have come to the conclusion that we should be able to consider a revised version of this manuscript in which you need to address the criticisms raised by referees 1 and 2 by further experimentation in an adequate manner and to their satisfaction. It will be indispensable to provide stronger evidence for the physiological significance of your findings in primary neurons along the lines suggested. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS
Referee #1 (Remarks to the Author):

The authors provide evidence that ADAM10, but not the closely related ADAM9 and ADAM17 proteases, cleave APP in cultured primary neurons. Interestingly, the authors show that when ADAM10 is depleted from the neurons (by RNAi) there is not an increase in beta-secretase cleavage of APP. This is an important area of investigation and the authors' data are novel and a valuable contribution to the fields of cell biology and neuroscience.

1. One concern is that the authors perform an excellent complementary set of analyses of APP metabolism with various genetic and pharmacological manipulations of secretases in cultured tumor cell lines. Collectively, the data clearly show that ADAM10 Is the key alpha-secretase in the cell lines. However, they only perform one experiment (using KDADAM10) to evaluate the role of ADAM10 as an alpha-secretase in primary neurons. The claim In the Title that ADAM10 is the physiologically relevant alpha-secretase in primary neurons is therefore not fully justified. Roles for other ADAMs in primary neurons were not evaluated.

2. The authors did not measure a biological enpoint, that is a response of cells to sAPPalpha, in neurons in which ADAM10, ADAM17 or ADAM9 are knocked down. This would seem important.

Referee #2 (Remarks to the Author):

The authors examined the physiological relevance of ADAM10 on alpha-cleavage of APP using a novel antibody that specifically recognizes human APPsalpha. They showed that knockdown of ADAM10, but not ADAM9 or 17, selectively decreased APPsalpha from HEK293, SH-SY5Y and primary neurons from APP transgenic mice. They also determined the cleavage site by ADAM10, which corresponded to the conventional alpha-cleavage site of APP. Inhibition of ADAM10 or BACE1 did not cause an increase in the APPsbeta or alpha, respectively. They concluded that ADAM10 is the constitutive alpha-secretase of APP.
The results of this study is largely consistent with those of previous reports, in that fibroblasts from ADAM10 knockout mice showed significant reduction in secreted APP, while significant variability in cells-to-cells has been reported. In this MS, the authors claim that the novel antibody 4B4 is able to discriminate APPsalpha and APPsbeta', the latter having confused results of previous studies. However, there remain a couple of important problems in terms of the physiological significance of ADAM10 on alpha-cleavage of APP.
1) Physiological relevance of ADAM10 in neurons: The title says "the role of ADAM10 in primary neurons", but only one figure (fig. 4) describes data obtained in primary neurons. In addition, only data on ADAM10 knockdown are shown in figure 4. The effect of RNAi on ADAM9 and 17 in primary neurons should be shown. Moreover, even in SY-5Y cells, ADAM10 knockdown failed to cause a complete loss of APPsalpha (e.g., fig. 4, 6). Experiments using neurons from ADAM10 knockout mice should be obtained.
2) Levels of APPsalpha, APPsbeta and APPsbeta': The absolute levels of secreted APP species are quite important issues in this study. The authors discuss quantitatively the contribution of ADAM10 and BACE1 on the secretion of APP in baseline condition. In fact, 40% of total APPs remained upon TAPI treatment and ADAM10 knockdown in fig. 3B. Does BACE1 contribute to APP secretion at such a high level? Does double treatment with TAPI and C3 totally abolish the total APPs?
3) ADAM10 in AD pathophysiology: The authors claim that C99, a direct precursor of Abeta, is processed and degraded by ADAM10 to generate C83. The authors should show the changes in Abeta production by ADAM10 knockdown. Using primary neurons expressing APP in fig. 4 should

enable the detection of Abeta. Indeed, in a therapeutic context, ADAM10 would not be a good drug target; rather, ADAM17 should be a better target, because the activity of ADAM17 is "regulated" by cellular signaling. In fact, several lines of data indicate that the activation of alpha-secretase activity lowers Abeta generation. The authors should discuss this issue more carefully.

Referee #3 (Remarks to the Author):

This manuscript addresses a longstanding controversy in the Alzheimers Disease field regarding the identity of the major alpha secretase for the Amyloid Precursor Protein (APP). A proteolytic breakdown product of the APP, the A-beta peptide, is widely considered to be the critical causative factor in the pathogenesis of this devastating disease. The A-beta peptide is generated through sequential processing of APP by the beta- and gamma-secretase, and once released from the precursor molecule, it has a propensity to aggregate and give rise to amyloid plaques. However, if APP is processed first by a different enzyme, termed alpha secretase, this prevents the production of A-beta, and so the alpha secretase is considered a protective enzyme in the context of Alzheimer's disease. Therefore there has been considerable interest in identifying the physiologically relevant alpha secretase in neuronal cells. Previous studies have implicated several membrane-anchored metalloproteinases of the ADAM (a disintegrin and metalloproteinase) family as alpha secretases, including ADAM9, ADAM10 and ADAM17, and recent reviews in this area cite these three ADAMs as candidate alpha secretases.

The current study by Dr. Lichtenthaler's group provides unambiguous evidence that ADAM10 is the major constitutive alpha secretase in different neuronal cell types, and that the PMA-stimulated alpha secretase is ADAM17. This clearly delineates which enzyme does what, and also rules out the frequently invoked compensatory functions of these two enzymes. When ADAM10 is knocked down, ADAM17 cannot compensate with respect to constitutive alpha-secretase activity, whereas the lack of ADAM10 does not affect the PMA stimulated alpha secretase activity. So, even though these two ADAMs have been implicated as alpha secretases before, this side-by-side comparison clearly delineates the precise contribution of each enzyme under different conditions. Moreover, the authors show that ADAM9 has no detectable role as alpha secretase under the conditions used in this study. The data are of the highest quality, and a monoclonal antibody that specifically recognizes the neoepitope generated by alpha-secretase cleavage (developed by this group) helps to clearly distinguish alpha secretase activity from the recently reported APP-beta' processing by beta secretase at a closely adjacent site. All appropriate controls are included in each figure, and the discussion is completely supported by the results. In addition to identifying the relevant APP alpha secretase, the authors also address other important questions in the field of APP processing. They show that there is no competition between constitutive alpha and beta secretase-dependent processing of APP, arguing against the widely held belief that blocking one could increase processing by the other. They also provide compelling evidence for the concept that the membrane-anchored stub of APP that is generated by beta secretase can then be subjected to alpha secretase processing. Even though this is only seen when gamma secretase is inhibited, the authors provide an excellent discussion of the potential significance of this finding, cite other papers in support of its relevance, but also discuss all caveats.

Overall, this is an outstanding manuscript that lays to rest a longstanding controversy in the literature regarding the identity of the physiologically relevant APP alpha secretase in neuronal cells. This manuscript will be of significant interest to researchers in the Alzheimers field, and to those studying cell surface proteolysis, matrix metalloproteinases, cell-cell interactions and how to understand the contribution of various candidate enzymes to a disease process. In this reviewer's opinion, this manuscript is suitable for publication in the EMBO Journal as it is, without any further revisions

1st Revision - authors' response 25 June 2010

Reviewer 3
Thank you very much for your review. No further experiments or changes needed to be done.

Reviewer 1

1. One concern is that the authors perform an excellent complementary set of analyses of APP metabolism with various genetic and pharmacological manipulations of secretases in cultured tumor cell lines. Collectively, the data clearly show that ADAM10 Is the key alpha-secretase in the cell lines. However, they only perform one experiment (using KDADAM10) to evaluate the role of ADAM10 as an alpha-secretase in primary neurons. The claim In the Title that ADAM10 is the physiologically relevant alpha-secretase in primary neurons is therefore not fully justified. Roles for other ADAMs in primary neurons were not evaluated.

We carried out the suggested experiment and knocked-down ADAM9 and ADAM17 in primary neurons. In agreement with the results obtained in the cell lines, we found that ADAM9 and ADAM17 do not affect alpha-secretase cleavage of APP. The data, including the measurement of APPsbeta, are shown in new figure 6 and described in the results' section. Together, our experiments demonstrate that among the three candidate alpha-secretases ADAM9, 10 and 17 only the knock-down of ADAM10 led to a nearly complete loss of alpha-secretase cleavage, demonstrating that ADAM10 is essential for alpha-secretase cleavage in primary neurons. The remaining few percent of alpha-secretase cleavage product correlate with the remaining few percent of ADAM10 protease being expressed in the knock-down neurons. In the initial version of the manuscript we had used neurons from APP overexpressing I5 mice. However, the mice stopped producing offspring for an unknown reason. Thus, we changed to wild-type mice, which has the additional advantage that we are now looking at the endogenous APP and its cleavage products. However, this required us to generate a new monoclonal antibody, which detects murine APPsalpha with good sensitivity. The available human APP antibodies could not be used, as there is an amino acid change between the human and the murine APP sequence close to the alphasecretase cleavage site. Our new monoclonal antibody (7A6) is described in Fig. 1.

2. The authors did not measure a biological enpoint, that is a response of cells to sAPPalpha, in neurons in which ADAM10, ADAM17 or ADAM9 are knocked down. This would seem important.

We fully agree with the reviewer and would love to show a function for APPsalpha. However, the biological function of APPsalpha is not yet clearly established. From in vivo experiments it is clear that APPsalpha does have a biological function. Transgenic expression of APPsalpha in APP knock-out mice rescues the mild phenotype of APP knock-out mice, including reduced body weight and reduced grip strength (Ring et al. J Neurosci 2007). These in vivo experiments suggest a role for APP in the function of neurons, but the underlying molecular mechanisms and functions of APP remain to be determined in these mice. Additionally, several studies in the past addressed the function of APP using in vitro and in vivo assays and reported for example that APPsalpha is neuroprotective and is increasing the length of neurites. We tested for these functions, as suggested by the reviewer. We expected to see changes, depending on whether APPsalpha was generated or not. In agreement with a previous publication (Furukawa et al. J. Neurochem 1996), we used glutamate or NMDA to induce cell toxicity in primary neurons and in neuroblastoma SH-SY5Y cells. As a readout for cell toxicity we used three different assays (trypan blue staining or Topro3 staining or the MTT assay). We had expected to observe an enhanced toxicity in ADAM10 knockdown cells compared to the wild-type cells – resulting from the reduced generation of the neuroprotective APPsalpha in the ADAM10 knock-down cells. However, we did not observe any reproducible difference between wildtype and ADAM10 knock-down cells. Changes in compound concentration or experiment duration did also not lead to clear changes between wild-type and ADAM10 knock-down cells. Likewise, addition of recombinant APPsalpha (obtained from two different companies) did not result in significant changes in the toxicity between wild-type and ADAM10 knock-down cells. This lack of a functional change may be explained in the following manner: Besides APP, ADAM10 has an increasing number of substrates, many of which are also expressed in neurons and neuronal cells. Similar to APP, these substrates undergo less secretion in ADAM10 knock-down cells, which in turn may affect the cellular stress response positively or negatively. Thus, we conclude that the use of ADAM10 knock-down cells is unlikely to be a good system to measure a molecular function of APP, which is not yet very well established. A similar conclusion was drawn in a recent study (Clement et al Neurosci 2008), which investigated the effect of neuronal ADAM10 modulation in an in vivo model of acute excitotoxic stress. Under some conditions ADAM10 activity provided a neurotrophic effect, whereas under other conditions it enhanced neurotoxicity or had no effect. The authors concluded that "additional cleavage products

of ADAM10 may counterbalance the neuroprotective effect of alpha-secretase-cleaved APP in the defense against excitotoxicity".

Other previous studies had concluded that APPsalpha is able to influence the length of neurites in neurons (e.g. Small et al. J Neurosci 1994, Young-Pearse et al. Neural Dev 2008, Gakhar-Koppole et al. Eur. J Neurosci 2008). However, the opposite conclusion was drawn in a recent study (Bergmans et al. Stem Cells 2010). Thus, the function of APPsalpha in controlling neurite length remains unclear. Nevertheless, we measured the neurite length of embryonic neurons in culture, which were transduced with control vector or shRNAs against the ADAM proteases. We detected a trend to a reduced neurite length in the ADAM10 knock-down neurons compared to the control treated neurons, which would be in agreement with a role of APPsalpha in promoting neurite length. However, this change was not statistically significant. Thus, we conclude that also in this experimental setting the ADAM10 knock-down is not appropriate to measure changes of APPsalpha function.

In summary, we conclude that we still do not know enough about APPsalpha function and its possible functional cross-talk with other ADAM10 substrates, in order to observe functional changes of APPsalpha in ADAM protease knock- down cells.

Reviewer 2

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We carried out the suggested experiment and knocked-down ADAM9 and ADAM17 in primary neurons. In agreement with the results obtained in the cell lines, we found that ADAM9 and ADAM17 do not affect alpha-secretase cleavage of APP. The data, including the measurement of APPsbeta, are shown in new figure 6 and described in the results' section.
Additionally, we generated new shRNAs against ADAM10, which allowed us to obtain a much better knock-down efficiency of ADAM10 in neurons compared to the previous version of the manuscript (new figure 6). With the new ADAM10 shRNAs the protein expression level of ADAM10 is less than 10% and thus so low that it is similar to a knock-out. This is also reflected by the finding that the alpha-secretase cleavage product of APP (APPsalpha) was reduced to less than 10%.
A similar reduction of ADAM10 expression and alpha-secretase cleavage to about 10% was observed in the experiments using the SH-SY5Y cells: see Fig. 4A, B and APP-TEV-FLAG experiment in new figure 7 (previously figure 6), in particular for the sh9 knock-down construct of ADAM10.
Together, our experiments demonstrate that among the three candidate alpha-secretases ADAM9, 10 and 17 only the knock-down of ADAM10 leads to a nearly complete loss of alpha-secretase cleavage. The remaining few percent of alpha-secretase cleavage product correlate with the remaining few percent of ADAM10 protease in the knock-down cell lines and neurons.

In the initial version of the manuscript we had used neurons from APP overexpressing I5 mice. However, the mice stopped producing offspring for an unknown reason. Thus, we changed to wild-type mice, which has the additional advantage that we are now looking at the endogenous APP and its cleavage products. However, this required us to generate a new monoclonal antibody, which detects murine APPsalpha with good sensitivity. The available human APP antibodies could not be used, as there is an amino acid change between the human and the murine APP sequence close to the alphasecretase cleavage site. Our new monoclonal antibody (7A6) is described in Fig. 1.

2) Levels of APPsalpha, APPsbeta and APPsbeta': The absolute levels of secreted APP species are quite important issues in this study. The authors discuss quantitatively the contribution of ADAM10 and BACE1 on the secretion of APP in baseline condition. In fact, 40% of total APPs remained upon TAPI treatment and ADAM10 knockdown in fig. 3B. Does BACE1 contribute to APP secretion at such a high level? Does double treatment with TAPI and C3 totally abolish the total APPs?

We carried out the suggested experiment. Double treatment with TAPI plus C3 does reduce total APP secretion further than the individual inhibition of a-(TAPI) or b-secretase (C3) cleavage alone (new Fig. 4E). For example, Figure 4C (and the quantification in 4D) shows that the stable knock-down of ADAM10 reduces total APP secretion in SH-SY5Y cells to about 40%. Additional inhibition of b-secretase with the specific inhibitor C3 further reduced total APP secretion to 20%. This shows two things. First, b-secretase cleavage contributes to about 20% to total APP shedding in SH-SY5Y cells. This pharmacological experiment is confirmed by a genetic experiment in HEK293 cells, where the knock-down of the b-secretase BACE1 reduced total APP shedding by 20% (new Fig. 3H). Second, the inhibition of b-secretase in the ADAM10 knock-down cells demonstrates, that a- and b-secretase make up at least 80% of total APP secretion, leaving at most 20% for other proteases to cleave APP. Within the range of experimental variation (see error bars) this is in good agreement with the double inhibition (TAPI plus C3, Fig. 4E), which reduced total APP secretion to about 10%. This suggests that an additional protease may contribute to APP shedding, to about 10%. In fact, a previous study provided evidence for a putative delta-secretase cleavage of APP at a position 12 amino acids upstream of the b-secretase cleavage site (Simons et al. J. Neuroscience 1996). Such a cleavage product would be detected by the N-terminal APP antibody 22C11, which we used. However, given that a delta-secretase has not yet been identified, it is unclear whether the about 10% remaining APP secretion in our experiment are indeed due to a putative delta-secretase cleavage or due to another as yet unidentified protease cleaving in the vicinity.

3) ADAM10 in AD pathophysiology: The authors claim that C99, a direct precursor of Abeta, is processed and degraded by ADAM10 to generate C83. The authors should show the changes in Abeta production by ADAM10 knockdown. Using primary neurons expressing APP in fig. 4 should enable the detection of Abeta. Indeed, in a therapeutic context, ADAM10 would not be a good drug target; rather, ADAM17 should be a better target, because the activity of ADAM17 is "regulated" by cellular signaling. In fact, several lines of data indicate that the activation of alpha-secretase activity lowers Abeta generation. The authors should discuss this issue more carefully.

As suggested, we measured Abeta levels in control and ADAM10 knockdown cells, both in HEK293 cells and in primary neurons. In both cases, the endogenous Abeta was measured and was found to be unchanged in the HEK293 cells and mildly increased in the neurons upon ADAM10 knockdown. These results are included in Fig. 3 (HEK293 cells) and Fig. 6 (neurons). Given that Abeta levels were largely unchanged we toned down the paragraph in the discussion where we had speculated about the conversion of C99 to C83. We discuss this as a possibility, but clearly state that this seems to occur in particular, when g-secretase cleavage is blocked. Under normal conditions (without g-secretase inhibition) we state that the predominant processing of C99 occurs by g-secretase and not by asecretase. This part of the discussion is found on page 17, second paragraph. Regarding the regulated and constitutive a-secretase we changed and restructured the discussion as suggested and now discuss more carefully that the activation of a-secretase is a means to lower Abeta generation. This part of the discussion is found on pages 16-17. The final sentence concludes: “This shows that the regulated component of a-secretase (i.e. the increase of a-secretase cleavage above its constitutive level) can compete with b-secretase and consequently reduces Ab generation, in agreement with the idea that a pharmacological activation of a-secretase may be a therapeutic approach to AD”.

2nd Editorial Decision 28 June 2010

Thank you for sending us your revised manuscript. Our original referees 1 and 2 have now seen it again, and you will be pleased to learn that in their view you have addressed all criticisms in a satisfactory manner. The paper will now be publishable in The EMBO Journal and you will receive a formal acceptance letter shortly.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor

The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have addressed my concerns adequately.

Referee #2 (Remarks to the Author):

The authors have addressed all the concerns raised by referees, and the manuscript is suitable for publication in its present form.

